Answers to questions about

Proteases for Fusion Tag Removal

For some applications, it may be desirable to remove a fusion tag from a recombinant target protein during or after purification. Many expression vectors encode protease cleavage sites between the cloning sites and the fusion tag sequences for this purpose. We offer a variety of proteases and, for removal of protease following digestion, protease capture kits. However, choosing the best protease system for your needs depends on a number of factors. Here is some useful information to aid your selection.

I need to select an expression vector with appropriate protease cleavage sites. How can I easily compare the different proteases?

We offer several proteases for fusion tag cleavage, including HRV 3C, restriction grade thrombin and biotinylated thrombin, enterokinase, and Factor Xa. Table 1 summarizes the features of each type of protease.

When determining a protease cleavage strategy, consider the following factors: (see next page)

Table 1. Proteases used for fusion tag removal

<table>
<tr><th>Enzyme</th><th>Cleavage Site</th><th>Size</th><th>Specificity</th><th>Method of Enzyme Removal by Affinity Capture</th><th>Purified from</th><th>Recommended Cleavage Buffer Composition (1X)*</th><th>Recommended Cleavage Reaction Conditions**</th></tr>
<tr><td>Tag•off™ High Activity rEK (recombinant, human)</td><td>AspAspAspAspLys↓</td><td>26 kDa (calculated), 33 kDa (apparent)</td><td>High</td><td>EKapture™ Agarose >99% efficiency</td><td rowspan="2">E. coli expression host strain</td><td rowspan="2">50 mM NaCl, 20 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.4</td><td rowspan="10">Room temperature (20–23°C) 16 hr</td></tr>
<tr><td colspan="5">1 Unit of Tag•off High Activity rEK cleaves >95% of 50 µg Cleavage Control Protein in 1X rEK Cleavage/Capture Buffer at room temperature for 16 h.</td></tr>
<tr><td>Recombinant Enterokinase (rEK) (recombinant, bovine)</td><td>AspAspAspAspLys↓</td><td>26 kDa (calculated), 33 kDa (apparent)</td><td>High</td><td>EKapture™ Agarose >99% efficiency</td><td rowspan="2">E. coli expression host strain</td><td rowspan="2">50 mM NaCl, 20 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.4</td></tr>
<tr><td colspan="5">1 Unit of Recombinant Enterokinase (rEK) cleaves >95% of 50 µg Cleavage Control Protein in 1X rEK Cleavage/Capture Buffer at room temperature for 16 h.</td></tr>
<tr><td>Thrombin, Restriction Grade*** (human)</td><td>LeuValProArg↓GlySer</td><td>33 kDa (B chain) (A chain, 6 kDa)</td><td>Moderately High</td><td></td><td rowspan="4">Human plasma that tests negative for HBsAg and HIV antibodies</td><td rowspan="4">150 mM NaCl, 20 mM Tris-HCl, 2.5 mM $CaCl_2$, pH 8.4</td></tr>
<tr><td colspan="5">1 Unit of Thrombin cleaves 1 mg of Cleavage Control Protein in 1X Thrombin Cleavage Buffer at 20°C for 16 h.</td></tr>
<tr><td>Biotinylated*** Thrombin (human)</td><td>LeuValProArg↓GlySer</td><td>35 kDa (B chain) (A chain, 6 kDa)</td><td>Moderately High</td><td>Biotin / Streptavidin Agarose >99% efficiency</td></tr>
<tr><td colspan="5">1 Unit of Biotinylated Thrombin cleaves 1 mg of Cleavage Control Protein in 1X Thrombin Cleavage Buffer at 20°C for 16 h.</td></tr>
<tr><td>Factor Xa*** (bovine)</td><td>IleGluGlyArg↓</td><td>2 subunits 34, 29 kDa</td><td>Moderate</td><td>Xarrest™ Agarose >95% efficiency</td><td rowspan="2">Bovine plasma</td><td rowspan="2">100 mM NaCl, 50 mM Tris-HCl, 5 mM $CaCl_2$, pH 8.0</td></tr>
<tr><td colspan="5">1 Unit Factor Xa cleaves >95% of 50 µg Cleavage Control Protein in 1X Factor Xa Cleavage/Capture buffer at room temperature for 16 h.</td></tr>
<tr><td>HRV 3C Protease (recombinant, human rhinovirus)</td><td>LeuGluValLeuPheGln↓GlyPro</td><td>22 kDa</td><td>Very High</td><td>His•Tag® /Ni-NTA His•Bind® Resin >95% efficiency</td><td rowspan="2">E. coli expression host strain</td><td rowspan="2">150 mM NaCl, 50 mM Tris-HCl, pH 7.5</td><td rowspan="2">4°C 16 h</td></tr>
<tr><td colspan="5">1 Unit of HRV 3C Protease cleaves >95% of 100 µg HRV 3C Cleavage Control Protein in 1X HRV 3C Protease Cleavage Buffer at 4°C for 16 h.</td></tr>
</table>

* 10X Cleavage or Cleavage/Capture Buffers included with all protease kits.
**In general, for all proteases, cleavage reaction temperature range is 4–37°C and incubation time range is 2–16 h.
*** For a review comparing thrombin and Factor Xa cleavage reaction methods, see: Jenny, R.J, Mann, K.G., and Lundblad, R. L. 2003. *Prot. Exp. Purif.* **31**, 1–11.

- Verify that the protease recognition site is not present within your protein of interest.
- Determine whether your downstream application requires removal of all vector-encoded amino acids. Factor Xa, rEK, and Tag•off™ rEK cleave at the carboxy-terminus of their recognition sequences, which (depending on cloning strategy) may result in complete removal of extraneous amino acids. Thrombin and HRV 3C protease each cleave at internal sites.
- Decide whether the protease itself needs to be removed from the reaction following digestion. If so, determine whether the removal process (see Table 1) will be compatible with your protein of interest. For example, target proteins bearing a C-terminal His•Tag® sequence are incompatible with removal of HRV 3C protease by immobilized metal affinity chromatography (IMAC). EKapture™ Agarose and Xarrest™ Agarose are not appropriate to remove rEK and Factor Xa (respectively) when the target protein is a serine protease, because these purification systems are based on affinity to serine proteases.
- Depending on the scale of your project (see Table 2), cost may be a consideration. Customers conducting large-scale protease cleavage reactions are welcome to contact Bulk and Custom Services. For assistance and price quotes, call (800) 854-2855 or e-mail custom.services@emdbiosciences.com.

In short, as with cloning strategy, it is beneficial to determine a protease cleavage strategy from the beginning of any recombinant protein project.

Table 2. Relative units of proteases needed to cleave 50 µg control protein

Protease	Units needed to digest 50 µg control protein
Tag•off High Activity rEK	1 U
rEK	1 U
Thrombin, Restriction Grade	0.05 U
Biotinylated Thrombin	0.05 U
HRV 3C	0.5 U
Factor Xa	1 U

After the cleavage reaction incubated overnight in cleavage buffer, the cleavage control protein digested correctly, but the target protein did not. Why?

The protease cleavage site may not be fully exposed in the folded fusion protein. It may be helpful to add a detergent or denaturant that is compatible with the protease. See Table 3 for the protease compatibility of several commonly used detergents, denaturants, and other reagents. The optimal level of denaturant should be tested empirically for each target protein prior to scaling up the digestion reaction.

I see too many low molecular-weight products. What's going on?

It is critical to determine whether these products appear pre-lysis, post-lysis, or during the protease reaction. Pre-lysis truncated products can accumulate due to endogenous bacterial proteases (using a protease-deficient expression strain such as BL21(DE3) or its derivatives is advisable), or incomplete translation due to rare codons (in this case, Rosetta™ (DE3), Rosetta 2 (DE3), or their derivatives may be useful). If degradation occurs post-lysis but prior to the protease reaction, this may be due to inherent target protein lability or presence of residual host strain proteases. Conducting purification at low temperature may help with the former, and use of protease inhibitors throughout target protein purification may help with the latter – provided that the inhibitors do not negatively impact the cleavage reaction. (In general, all Novagen® proteases are inhibited by serine protease inhibitors such as PMSF, AEBSF, and APMSF.) If, however, secondary cleavage occurs during the protease reaction, it may be possible to optimize reaction conditions to minimize this effect. In general, adding excess protease is inadvisable as this can promote secondary cleavage. Some commonly used agents may also promote secondary cleavage (see Table 3). Finally, if contaminating proteins are present in the target protein preparation, note that the contaminants themselves may have protease cleavage sites.

Can I digest my protein while it is still bound to the column?

In general, on-column cleavage is usually not as efficient as cleavage in solution, and thus may require higher amounts of protease per mg target protein. Test conditions empirically before scaling up. The User Protocol for HRV 3C protease (TB420) includes a detailed protocol for on-column digestion of target protein bound to Ni-NTA His•Bind resin. If using rEK or thrombin to cleave S•Tag™ target proteins, it is possible to perform the cleavage reaction while the protein is bound to S-protein agarose beads in batch mode. In the S•Tag Thrombin Purification Kit, biotinylated thrombin can be removed using streptavidin agarose (see User Protocol TB087). If using rEK, the protease can be removed with EKapture agarose (see User Protocol TB160).

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How can we help you?

Table 3. Effects of common reagents and on protease activity. Tinted blue boxes indicate a negative impact of the agent on protease activity level. For specific reagent concentrations, refer to the User Protocol for each protease. White boxes indicate no inhibition. ND, not determined.

Agent	Tag•off™ High Activity rEK	rEK	Biotinylated Thrombin	HRV 3C	Factor Xa
Detergents					
TRITON® X-100					
TWEEN® 20					
SDS				ND	
Denaturing & Reducing Agents					
Urea					
Guanidine	ND		ND		
DTT	*			See below	
Salts					
NaCl	**				
Elution agents					
Imidazole					
Notes	* compatible with up to 20 mM DTT ** compatible with up to 3 M NaCl - Active at pH 5–9	- Active at pH 5–9 - low levels of SDS (0.0625%) promote secondary cleavage	- Reduced activity at low pH (e.g., 20 mM MES pH 5.0 or 6.0) or low temperature (4°C) - Activity enhanced by heparin - compatible with 1 mM glutathione	- 100 mM $ZnCl_2$ causes 50% inhibition - Compatible with DTT, EDTA, EGTA; however, these agents are not recommended prior to protease removal using IMAC	- Activity partially inhibited by N-laurylsarcosine or NP-40. Low levels of SDS (≥0.001%) promote secondary cleavage. - Activity inhibited by ethylene glycol
For more information see User Protocol (available at www.novagen.com)	TB458	TB150	TB188	TB420	TB205

When working with a recombinant protein bearing a His•Tag® or GST•Tag™, can I perform the protease digestion on eluted protein directly?

In many cases, the elution conditions of the purification scheme are inhibitory to protease activity (see Table 3). Therefore, we recommend first eluting the target protein, and then dialyzing it into appropriate cleavage buffer prior to protease digestion. Maximum concentrations of imidazole tolerated by each protease are described in User Protocol documents.

What cleavage buffer conditions are optimal? What do I need to consider if I want to use a different buffer?

The composition of 1X Cleavage Buffer (or Cleavage/Capture Buffer) supplied with each kit is shown in Table 1. If your target protein requires a different buffer, consider carefully the reagent compatibilities indicated in Table 3. If substituting a different buffer, perform pilot experiments using the Control Protein included with each protease kit. It should be noted that $CaCl_2$ is included in several Cleavage Buffers to promote protease stability, but is not necessary for full activity of Tag•off™ rEK, rEK, or thrombin. If your target protein requires $CaCl_2$-free buffer, perform trial digestions to determine suitability. ■

Product	Size	Cat. No.	Price
Thrombin, Restriction Grade	50 U	69671-3	$84
Biotinylated Thrombin	50 U	69672-3	$179
Thrombin Cleavage Capture Kit	1 kit	69022-3	$216
Recombinant Enterokinase	50 U	69066-3	$106
Enterokinase Cleavage Capture Kit	1 kit	69067-3	$183
Tag•off™ High Activity rEK	50 U	71537-3	$107
Tag•off™ rEK Cleavage/Capture Kit	1 kit	71540-3	$185
Factor Xa, Restriction Grade, Bovine Plasma	400 U	69036-3	$84
Factor Xa Cleavage Capture Kit	1 kit	69037-3	$198
HRV 3C Protease	500 U	71493-3	$178